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Vaara, Martti

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# Structure–activity studies on polymyxin derivatives carrying three positive charges only reveal a new class of compounds with strong antibacterial activity

Martti Vaara [a,b,c,*], Timo Vaara [a], Jonathan M. Tyrrell [d]

[a] Northern Antibiotics Ltd., FI-02150 Espoo, Finland
[b] Division of Clinical Microbiology, Helsinki University Hospital, FI-00029 Helsinki, Finland
[c] Department of Bacteriology and Immunology, Helsinki University Medical School, FI-00014 Helsinki, Finland
[d] Department of Medical Microbiology & Infectious Disease, Institute of Infection & Immunity, UGW Main Building, Heath Park Campus, Cardiff, Wales, UK

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ABSTRACT

Recent years have brought in an increased interest to develop improved polymyxins. The currently used polymyxins, i.e. polymyxin B and colistin (polymyxin E) are pentacationic lipopeptides that possess a cyclic heptapeptide part with three positive charges, a linear "panhandle" part with two positive charges, and a fatty acyl tail. Unfortunately, their clinical use is shadowed by their notable nephrotoxicity. We have previously developed a polymyxin derivative NAB739 which lacks the positive charges in the linear part. This derivative is better tolerated than polymyxin B in cynomolgus monkeys and is, in contrast to polymyxin B, excreted into urine in monkeys and rats. Here we have conducted further structure-activity relationship (SAR) studies on 17 derivatives with three positive charges only. We discovered a remarkably antibacterial class, as exemplified by NAB815, that carries two positive charges only in the cyclic part.


## 1. Introduction

Recent years have brought in an increased interest to develop improved polymyxins for the therapy of severe infections caused by extremely antibiotic-resistant Gram-negative bacteria, as reviewed by Pirri et al. [10], Vaara [12], Kadar et al. [5] Cochrane and Vederas [2] and Velkov et al. [19].

The currently used polymyxins, i.e. polymyxin B and colistin (polymyxin E) were discovered as early as 1947. They are pentacationic lipopeptides that possess a cyclic heptapeptide part with three positive charges, a linear "panhandle" part with two positive charges, and a fatty acyl tail (methyloctanoyl in polymyxin B1 and colistin A, or methylheptanoyl in polymyxin B2 and colistin B). For the structure of polymyxin B, see Fig. 1.

Polymyxin B and colistin (administered as its inactive methanesulphonate prodrug CMS) are effective against most species of Enterobacteriaceae as well as *Acinetobacter baumannii* and *Pseudomonas aeruginosa* but both were largely abandoned in the sixties because of nephrotoxicity and increased availability of more effective antibiotics [4–6,12]. According to recent studies, the nephrotoxicity rate of polymyxin B and colistin varies from 20% to 60%. Furthermore, pharmacokinetic data indicates that the current dosage regimens are suboptimal [3]. Therefore, larger doses should be used, but this further increases the risk of nephrotoxicity.

We have previously developed polymyxin derivatives such as NAB739 (Table 1) which lack the positive charges in the linear part and accordingly carry three positive charges only [12–15]. In contrast to the old polymyxins that are effectively reabsorbed by the proximal tubular kidney cells and not excreted as a biologically active form into urine, NAB739 and compounds related to it yield very high concentrations in the urine [12,14,17]. This is remarkable, since most of the bacteremic infections caused by Enterobacteriaceae originate from complicated urinary tract infections. NAB739 has reduced affinity for the brush border membrane of the proximal tubular cells and was shown to be less toxic than polymyxin B to these cells by a factor of more than 20 [13,16]. Preclinical studies on NAB739 are ongoing.

* Corresponding author at: Northern Antibiotics Ltd., Technopolis, Tekniikantie 14, FI-02150 Espoo, Finland.
*E-mail address:* martti.vaara@northernantibiotics.com (M. Vaara).

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**Table 1**
Structure of the compounds, their activity against *E. coli*, and their toxicity to HK-2 cells.[a]

| Compound | R (FA) | R1 | R2 | R3 | R4 | R5 | R6 | R7 | R8 | R9 | R10 | MIC (μg/ml)[b] for *E. coli* AT25922 | MIC (μg/ml)[b] for *E. coli* IH3080 | $IC_{50}$ (μg/ml)[c] for HK-2 cells |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Polymyxin B | MOA/MHA | -Dab$^+$ | -Thr | -Dab$^+$ | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Thr] | **0,5 (0,5-1) [15]** | **0,5 (0,5-1) [15]** | 18,0 (7,6 - 38) [4] |
| NAB739 | OA | - | -Thr | -DSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Thr] | **2 (1-4) [30]** | **2 (1-4) [30]** | **237 (184-275) [3]** |
| NAB807 | OA | - | -Thr | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Thr] | 4 (2-4) [6] | 4 (4-8) [6] | **235** |
| NAB809 | OA | - | -Thr | -DHSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Thr] | 16 [3] | 16 (16-32) [3] | - |
| NAB743 | OA | - | -Thr | -Ser | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Thr] | ≥ 32 [3] | 16 [3] | **113** |
| NAB805 | OA | - | -Thr | Thr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Thr] | ≥ 32 [3] | ≥ 32 [3] | **221** |
| NAB803 | OA | - | -Thr | HSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Thr] | ≥ 32 [3] | ≥ 32 [3] | **317** |
| NAB813 | OA | -Dab$^+$ | -Thr | -DSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Abu | -Dab$^+$ | -Thr] | **2 [6]** | **2 [6]** | - |
| NAB819 | NA | -Dab$^+$ | -Thr | -DSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Abu | -Dab$^+$ | -Thr] | **2 [3]** | **2 [3]** | - |
| NAB812 | OA | -Dab$^+$ | -Thr | -DSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Ser | -Dab$^+$ | -Thr] | **2 [3]** | **2 (1-2( [3]** | - |
| NAB818 | OA | -Dab$^+$ | -Thr | -DSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Ser | -Dab$^+$ | -Thr] | **2 [3]** | **2 (1-2( [3]** | - |
| NAB815 | OA | -Dab$^+$ | -Thr | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Abu | -Dab$^+$ | -Thr] | **2 (2-4) [24]** | **2 (2-4) [24]** | **334** |
| NAB820 | NA | -Dab$^+$ | -Thr | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Abu | -Dab$^+$ | -Thr] | 4 (2-4) [3] | **2 [3]** | - |
| NAB814 | OA | -Dab$^+$ | -Thr | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Ser | -Dab$^+$ | -Thr] | **2 [3]** | **2 [3]** | - |
| NAB821 | OA | -Dab$^+$ | -Thr | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Leu | -Dab$^+$ | -Thr] | 4 (4-8) [3] | 4 (4-8) [3] | - |
| NAB816 | OA | - | -Dab$^+$ | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Abu | -Dab$^+$ | -Thr] | > 64 [3] | > 64 [3] | - |
| NAB817 | OA | - | -Thr | -Dap$^+$ | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Abu | -Dab$^+$ | -Thr] | 16 [3] | 8 [3] | - |
| NAB822 | OA | -Dab$^+$ | -Thr | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Abu | -Dab$^+$ | -Leu] | 64 (64 - >64) [3] | 8 [3] | - |
| NAB823 | OA | -Dab$^+$ | -Thr | -DThr | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Leu | -Dab$^+$ | -Leu] | 16 (16-32) [3] | 8 [3] | - |
| NAB824 | OA | - | -Thr | -DSer | -cy[Dab | -Dab$^+$ | -DPhe | -Leu | -Dab$^+$ | -Dab$^+$ | -Leu] | 32 (16-32) [3] | 16 [3] | 56 |

[a] *Abbreviations:* MOA/MHA, a mixture of methyloctanoic and methylheptanoic acid; OA, octanoic acid; NA, nonanoic acid; Abu, aminobutyric acid; Dab, diaminobutyric acid; Fser, homoserine; cy indicates the R4-R10 ring.
[b] Median, range (in parentheses), number of determinations (in brackets). Median values <4 in **bold**.
[c] Geometric mean, range (in parentheses), number of determinations (in brackets). Values >100 in **bold**.

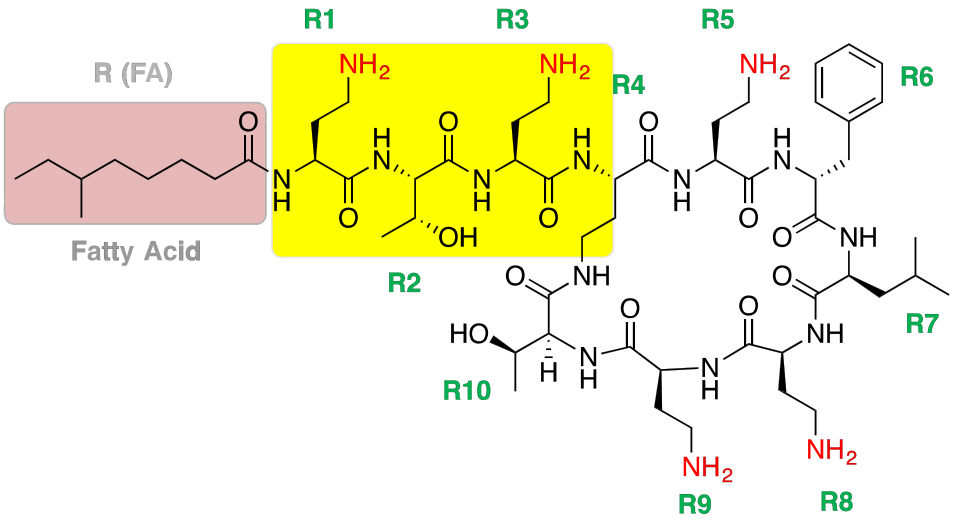


**Fig. 1.** The structure of polymyxin B1. The fatty acyl "tail" is highlighted in pink and the linear peptide part in yellow. The five free amino groups are marked in red. Polymyxin B2 carries 6-methyl heptanoic residue as the fatty acyl tail.

NAB739 lacks the residue R1 (diaminobutyric acid, Dab) and carries DSer at the position R3 instead of Dab (residue numbering according to that of polymyxin B and colistin with residues R1–R10). In structure–activity relationship (SAR) studies, R3 is one of the last "uncharted" positions. We postulated that in derivatives with three positive charges only, a hydrophilic residue carrying a hydroxyl group at R3 is advantageous for antibacterial activity, since a derivative identical to NAB739 but carrying aminobutyric acid (Abu) at R3 lacks direct antibacterial activity. In this communication, we have compared the activity of NAB739 with our newer derivatives that carry certain other residues than D-Ser at R3. Furthermore, our previous studies have given indirect evidence that in the ring part the positive charge at R8 due to a Dab residue is less important for the antibacterial activity than the Dab residues at R5 and R9. Here we show that it is indeed possible to design directly antibacterial derivatives with three positive charges only but no more than two of them in the ring part.

## 2. Materials and methods

### 2.1. Peptides

Milligram-scale synth of polymyxin derivatives was performed by conventional solid-phase chemistry at Alta Bioscience, Birmingham, United Kingdom, as described previously [13]. Briefly, the α-amino group was protected by fluorenylmethoxycarbonyl (Fmoc), the γ-amino group of the Dab residue involved in cyclization of the peptide by *t*-butoxycarbonyl (tBoc), and all the other functional side chain groups by benzyloxycarbonyl (Z). Acylation was performed for 30 min by using a 4-fold molar excess of each amino acid or the fatty acid. The cyclization mixture was added to the peptide (dissolved in dimethylformamide) and allowed to react for 2 h. The cyclized, protected peptide was precipitated by the addition of cold diethyl ether and washed with water. The remaining side chain protection groups (Z) were removed by catalytic hydrogenation as described previously [13]. The peptides were purified by HPLC using conventional gradients of acetonitrile–water–trifluoroacetic acid and the eluate fractions corresponding to the peptides were lyophilized. The peptide purity, estimated by HPLC, was more than 95%. All peptides were studied in their trifluoroacetate salt form.

Gram-scale synthesis of NAB739 sulfate and NAB815 sulfate was performed by Bachem AG (Bubendorf, Switzerland). The purity, as estimated by HPLC, was 97.3% for NAB739 and 98.4% for NAB815. Polymyxin B sulfate was from Sigma–Aldrich (St. Louis, MO, USA; product number PO972).

### 2.2. Bacterial strains

Seven ATCC strains were used: *Escherichia coli* ATCC25922, *Klebsiella pneumoniae* ATCC13883, *A. baumannii* ATCC19606, *P. aeruginosa* ATCC27853, *Staphylococcus aureus* ATCC25923, *S. aureus* ATCC29123, and *Enterococcus faecalis* ATCC29112. *E. coli* IH3080 (K1:O18:H7) is a virulent encapsulated strain [13,18], and *E. coli* SC 9253 is a well-characterized polymyxin-resistant *pmrA* mutant of *E. coli* K-12 [8,9,13]. Both strains have widely been used in our previous studies. The *tolC* construct of *E. coli* ATCC25922, the *E. coli* K12 strain D22 and *P. aeruginosa* PAO1 are from the University of Uppsala strain collection (Uppsala, Sweden). The clinical isolates of *E. coli* ($n = 111$), *K. pneumoniae* ($n = 89$) and *A. baumannii* ($n = 52$) are from the Innovative Medicines Initiative (IMI) ENABLE collection, preserved at the University of Uppsala and comprise strains from Cardiff (Wales, UK), Copenhagen (Denmark), Uppsala (Sweden) and Warsaw (Poland). *A. baumannii* strains EN117, EN118, EN151 and EN152 are from the IMI ENABLE collection.

### 2.3. Antibacterial assays

MIC determinations employing the broth microdilution method were according to CLSI protocol M07-A10 [1] and used microtiter plates, cation-adjusted Mueller–Hinton II broth (Difco 212322), and an inoculum size of $5 \times 10^5$ CFU/ml.

MIC determinations using the agar dilution method also were according to CLSI protocol M07-A10 [1]. Mueller–Hinton agar (Becton Dickinson, Oxford, UK) plates containing increasing concentrations (0.125–64 μg/ml in 2-fold increments) of the test substances were used. The agar plates were inoculated (1 μl per spot, corresponding to approximately $10^4$–$10^5$ CFU/spot) using a multipoint inoculator and were incubated for 16–18 h at $35 \pm 2$ °C.

The synergism with rifampin (Sigma–Aldrich) was studied in microtiter plates as previously described using the fixed rifampin concentration of 0.5 μg/ml.

### 2.4. Toxicity to human kidney proximal tubular cell line HK2

The HK-2 cell line (CRL-2190TM; American Type Culture Collection, Manassas, VA, USA) is an immortalized proximal tubule cell line derived from a normal human kidney. The assay was performed as described in [16]. Cells were cultured in flasks (150 $cm^2$) in 25 ml of Keratinocyte Serum Free Medium (Invitrogen, Life Technologies Ltd., Paisley, UK) supplemented with human recombinant epidermal growth factor (5 ng/ml) and bovine pituitary extract (50 ng/ml) under standard conditions (37 °C in 5% $CO_2$). Cultures were maintained at 70% confluence with a maximum of 25 passages. For the cytotoxicity assay, cells were seeded 1 day prior to treatment into sterile 96-well plates at a density of $7.5 \times 10^3$ cells/well. Cytotoxicity of the drugs was assessed over a nine-point concentration range, starting at a concentration of 1000 μg/ml and performing 1:2 serial dilutions. Each concentration was tested in triplicate. Negative controls (growth medium only) were added to each plate. The plates were incubated for 24 h, after which the CellTiter-BlueTM cell viability assay (Promega, Madison, WI, USA), which measures conversion of resazurin, was performed according to the manufacturer's instructions. Dose–response curves were plotted and the half maximal inhibitory concentration ($IC_{50}$) values were computed using GraphPad Prism™ software (GraphPad Software Inc., La Jolla, CA, USA).

## 3. Results

We first constructed four mimics of NAB739, each with a different hydroxyl-containing amino acid residue at the position R3, where NAB739 carries D-Ser (Table 1). Compound NAB807 with D-Thr had considerable activity, albeit weaker than that of NAB739, against the *E. coli* strains used in the screening (ATCC25922 and IH3080). NAB809 carrying D-homoserine (D-HSer) was the weakest out of these three compounds. We have previously shown [13] and we also verify it here that NAB743, with L-Ser at R3, is much less active than NAB739, with D-Ser at R3, as a directly antibacterial agent. In line with this, NAB805, with L-Thr at R3, and NAB803, with L-HSer at R3, did not possess direct antibacterial activity in the study conditions used here. However, and quite noteworthy, NAB743 has been shown to be very active as a permeabilizer compound [13], as are many other NAB compounds that lack the direct antibacterial activity [12–15]. Accordingly, a D-amino acyl at R3 is not a prerequisite for an agent with notable permeabilizing activity.

NAB739 is a nonapeptide and hence lacks the amino acid residue at R1 whereas polymyxin B and colistin do possess R1 and carry the positively-charged Dab at this position. Our next step was to construct tricationic decapeptide compounds where R1 is Dab but that are designed to lack one of the three positive charges in the ring portion (R4–R10). According to our previous studies, the Dab residue at R8 appears to be less important for the antibacterial activity than the Dab residues at R5 and R9 [13,15]. Hence, we introduced R1 with Dab, replaced Dab in R8 with the neutral residues aminobutyric acid (Abu), Ser or Leu, and preserved the hydroxyl-containing D-amino acid residue (D-Ser as in NAB739, or D-Thr as in NAB807) at R3. Altogether, seven compounds were synthesized, five with octanoyl and two with nonanoyl as the fatty acyl tail. Quite remarkably, all displayed MICs identical to or close to those of NAB739 (Table 1, *E. coli* strains as targets). A good representative for this class is NAB815, which has octanoyl-Dab-Thr-DThr as the linear part and Abu at R8 of the cyclic part.

To better understand the function of Dab as the terminal amino acid residue, we synthesized NAB816, which is otherwise identical to NAB815 but carries the dipeptide Dab-DThr as the linear peptide part, instead of the tripeptide Dab-Thr-DThr in NAB815. NAB816 was much less antibacterial than NAB815 (Table 1).

Pentacationic polymyxin compounds with the cationic diaminopropionic acyl (Dap) at R3 have been reported to have increased antibacterial activity [7]. The tricationic NAB817 which lacks R1 and carries Dap at R3 and Abu at R8 displayed activity against *E. coli* but it was weaker than NAB815 or NAB739 (Table 1).

Octapeptins are structurally very close to polymyxins but are octapeptides. They lack the residues R1–R2 of polymyxins and have a fatty acid tail that is two methylene units longer than that of polymyxins [8,11,12]. Furthermore, they carry Leu at R10 whereas polymyxins carry Thr. Accordingly, they are more hydrophobic than polymyxins and might even have a nonspecific action against all biological membranes [12]. Octapeptin A has been reported to be much more active than polymyxin B against polymyxin-resistant *E. coli* strain SC 9253 but, on the other hand, also against Gram-positive bacteria and eukaryotes [8]. We have previously shown that the lipid A part of the lipopolysaccharide (LPS) of SC 9253 is decorated by phosphorylethanolamine and 4-aminoarabinose, both masking the anionic phosphate/pyrophosphate groups that are the target of polymyxins [9]. Now we constructed NAB824, which is otherwise identical to NAB739 but carries Leu instead of Thr at R10 (Table 1). NAB824 was more active than NAB739 against *E. coli* SC 9253 (MIC medians 16 μg/ml and 64 μg/for NAB824 and NAB739, respectively), but its median MIC for polymyxin-susceptible *E. coli* strains was as high as 16–32 μg/ml. Therefore, while NAB824 can be regarded as equally active against polymyxin-susceptible and polymyxin-resistant strains, NAB739 is clearly superior against polymyxin-susceptible strains.

All the derivatives in Table 1 displayed MICs higher than 16 μg/ml for *P. aeruginosa* ATCC27853 and *S. aureus* ATCC25923. We have also previously shown [13] that the reduction of positive charges in the polymyxin molecule results in weakened activity against *P. aeruginosa*. The absence of the activity against *S. aureus* shows that the mode of action of the derivatives constructed is as specific as in polymyxin B or colistin.

We have previously shown that NAB739 is significantly less toxic than polymyxin B to human kidney proximal tubular cell line HK-2 [16]. Table 1 shows that, besides NAB739, all the new derivatives that were tested were also less toxic than polymyxin B to HK-2 cells. For instance, NAB815 was less toxic by a factor of approx. 20. However, NAB824, the derivative that displayed the best activity against the polymyxin-resistant *E. coli* strain SC 9253 (see above), was only approx. 3–4-fold less toxic than polymyxin B.

We then compared the MICs of NAB815 with those of NAB739 and polymyxin B using an extended set of bacterial strains (Table 2), including clinical isolates of *E. coli* ($n = 111$), *K. pneumoniae* ($n = 89$) and *A. baumannii* ($n = 52$). Based on the $MIC_{90}$ values, NAB815 was 2-fold less active against *E. coli* and *K. pneumoniae* and 2-fold more active against *A. baumannii* than NAB739. Both agents were somewhat less active than polymyxin B against these three species.

We have previously shown that NAB739 and rifampin are synergistic against *A. baumannii* strains [13,15]. Table 2 shows that an 8-fold decrease in the MIC of NAB739 and NAB815 for *A. baumannii* ATCC19606 was found in the presence of a sub-inhibitory concentration (0.5 μg/ml) of rifampin. The MIC of rifampin for this strain in the absence of polymyxin B was 4 μg/ml. For four clinical *A. baumannii* strains tested (EN117, EN118, EN151, EN152), the MIC of NAB815 and NAB739 decreased by factors of 2–4 and 4–8, respectively, in the presence of rifampin, whereas no decrease was found when polymyxin B was combined with rifampin.

## 4. Discussion

This paper showed that it is possible to design remarkably active antibacterial polymyxins that carry only two positive charges in the ring part (i.e. at the positions R5 and R9) and one positive charge

**Table 2**
MICs (μg/ml) of NAB815, NAB739, and polymyxin B.

| | NAB815 | NAB739 | Polymyxin B |
|---|---|---|---|
| *E. coli* ATCC 25922 | 1 | 0.5 | 0.25 |
| *E. coli* ATCC 25922, *tolC* efflux deficient construct | 1 | 0.5 | 0.25 |
| *E. coli* D22 OM permeability mutant | 0.5 | 1 | 0.25 |
| *E. coli*, clinical isolates (n = 111), $MIC_{90}$ | 4 | 2 | 1 |
| *K. pneumoniae* ATCC 13883 | 2 | 2 | 0.5 |
| *K. pneumoniae*, clinical isolates (*n* = 89), $MIC_{90}$ | 8 | 4 | 1 |
| *A. baumannii* ATCC 19606 | 4 | 4 | 0.5 |
| *A. baumannii* ATCC 19606 in the presence of rifampin (0.5 μg/ml)[a] | 0.5 [4] | 1 [8] | 0.5 [1] |
| *A. baumannii*, clinical isolates (*n* = 52), $MIC_{90}$ | 4 | 8 | 1 |
| *P. aeruginosa* PAO1 | 64 | 16 | 2 |
| *Staphylococcus aureus* ATCC 29213 | >64 | >64 | 64 |
| *Enterococcus faecalis* ATCC 29212 | >64 | >64 | 64 |

[a] Determined by broth microdilution. The MIC of the peptides in the absence of rifampin is shown in brackets.

in the linear part (at the position R1). Out of the several derivatives constructed, NAB815 was studied in more detail.

We have shown elsewhere that in cynomolgus monkeys a very significant portion of the dose of NAB815 and NAB739 is excreted into urine within 8 h after an intravenous infusion (0–8 h recovery) [17]. After the infusion of 8 mg/kg of NAB815, the 0–8 h recovery was 38%, 55%, and 88% of the dose (percentages given for each animal). The corresponding recovery rates for NAB739 were 20%, 91%, and 92% and those for polymyxin B 1%, 2%, and 2% [17]. The resulting concentrations of NAB815 and NAB739 in the urine were very high. After the infusion of NAB815 at 8 mg/kg, concentrations as high as 175, 225, and 260 μg/ml were found either in the 0–4 or 4–8 h sample (concentrations given for each animal). The corresponding concentrations for NAB739 were 80, 140, and 155 μg/ml and those for polymyxin B 7, 9, and 15 μg/ml [17]. The high concentrations in the urine explain why NAB815 was more effective than polymyxin B in the therapy of *E. coli* murine urinary infection [17]. Furthermore, both NAB815 and NAB739 are better tolerated than polymyxin B in cynomolgus monkeys [17]. Accordingly, the newly designed NAB815 as well as the previously described NAB739 both have significant advantages over polymyxin B, thus making them potentially more useful than the old polymyxins in the therapy of infections caused by multiresistant Gram-negative bacteria. Further studies will reveal their role in clinical medicine.

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